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(54) Titre: ADENOVIRAUX COMPRENANT DEUX GENES THERAPEUTIQUES: SUICIDE ET IMMUNOSTIMULANT

(57) Abstract

Novel adenovirus-derived viral vectors, preparation thereof, and use thereof in gene therapy. In particular, defective recombinant adenoviruses including two therapeutic genes, i.e. a suicidal gene and an immunopotentiating or tumour-suppressor gene, are disclosed.

(57) Abrégé

La présente invention concerne de nouveaux vecteurs viraux dérivés des adénovirus, leur préparation et leur utilisation en thérapie génique. Elle concerne plus particulièrement des adénovirus recombinants défectifs comprenant deux gènes thérapeutiques, le premier étant un gène suicide et le second un gène immunostimulant ou un gène suppresseur de tumeurs.

Adenovirals comprising two therapeutic genes: suicide and immunostimulant

The present invention relates to new viral vectors, their preparation and their use in gene therapy. It also relates to the pharmaceutical compositions containing the said viral vectors. More particularly, the present invention relates to recombinant adenoviruses as vectors for gene therapy.

Gene therapy consists in correcting a deficiency or an abnormality (mutation, aberrant expression and the like) by introduction of a genetic information into the affected organ or cell. This genetic information may be introduced either in vitro into a cell extracted from the organ, the modified cell then being reintroduced into the body, or directly in vivo into the appropriate tissue. In this second case, different techniques exist, among which are various transfection techniques involving complexes of DNA and DEAE-dextran (Pagano et al., J.Virol. 1 (1967) 891), of DNA and nuclear proteins (Kaneda et al., Science 243 (1989) 375), of DNA and lipids (Felgner et al., PNAS 84 (1987) 7413), the use of liposomes (Fraley et al., J.Biol.Chem. 255 (1980) 10431) and the like. More recently, the use of viruses as vectors for the transfer of genes emerged as a promising alternative to these physical transfection techniques. In this regard, different viruses have been tested for their capacity

to infect certain cell populations. In particular, the retroviruses (RSV, HMS, MMS and the like), the HSV virus, the adeno-associated viruses, and adenoviruses.

Among these viruses, the adenoviruses exhibit certain properties which are advantageous for use in gene therapy. In particular, they have a fairly broad host spectrum, are capable of infecting quiescent cells, do not become integrated into the genome of the infected cell, and have so far not been associated with major pathologies in man. The adenoviruses are viruses with linear double-stranded DNA of about 36 kb in size. Their genome comprises especially an inverted repeat sequence (ITR) at their end, an encapsidation sequence, early genes and late genes (cf. Figure 1). The principal early genes are the E1 (E1a and E1b), E2, E3 and E4 genes. The principal late genes are the L1 to L5 genes.

Given the abovementioned properties of adenoviruses, they have already been used for the transfer of genes in vivo. To this end, different adenovirus-derived vectors have been prepared, incorporating different genes (β-gal, OTC, α-1AT, cytokines and the like). In each of these constructs, the adenovirus has been modified so as to make it incapable of replication in the infected cell. Thus, the constructs described in the prior art are adenoviruses deleted of the regions E1 (E1a and/or E1b) and optionally E3 at the level of which a heterologous

DNA sequence is inserted (Levrero et al., Gene 101 (1991) 195; Gosh-Choudhury et al., Gene 50 (1986) 161).

The present invention relates to new adenovirus-derived vectors which are particularly efficient for gene therapy applications. More particularly, the present invention results partly from the demonstration that it is possible to incorporate several genes of interest into adenoviruses, and to obtain a substantial expression of these different genes in the infected cells. The present invention also results from the construction of adenoviral vectors capable of incorporating several therapeutic genes under conditions allowing their optimum expression. It further results from the demonstration of a synergistic effect of the vectors of the invention, linked to the co-expression, in the same target cell, of complementary therapeutic genes. The present invention thus provides viral vectors exhibiting therapeutic properties which are completely advantageous for their use in gene or cell therapy. In particular, the vectors of the invention exhibit properties which are completely advantageous for use in the treatment of pathologies with episodes of cell hyperproliferation (e.g. cancers or restenosis).

Accordingly the present invention provides a defective recombinant adenovirus characterized in that it comprises two therapeutic genes, the first being a suicide gene and the second an immunostimulant gene or a tumour-suppressor gene.

The applicant has indeed shown that the simultaneous co-expression of such genes in the same target cell produced a particularly advantageous therapeutic antitumour effect which is far greater than the effect obtained by means of these genes alone or separately introduced.

The therapeutic genes used within the framework of the present invention may be a cDNA, a genomic DNA (gDNA) or a hybrid construct consisting, for example, of a cDNA into which one or more introns would be inserted. They may also be synthetic or semisynthetic sequences. In a particularly advantageous manner, a cDNA or a gDNA is used.

The two therapeutic genes incorporated into the adenoviral vectors according to the present invention may be arranged in different ways.

They may first of all constitute a single transcriptional entity under the control of a single promoter. In this configuration, the two genes are continuous and give rise to a single premessenger RNA. This arrangement is advantageous since it makes it possible to use only one transcriptional promoter.

The two therapeutic genes may also be placed under the control of separate transcriptional promoters. This configuration makes it possible to obtain higher expression levels, and offers a better control of expression of the genes. In this case, the two therapeutic genes may be inserted in the same orientation or in opposite orientations, into the same site of the denovirus genome or into different sites.

As suicide gene, the genes whose expression products confers on the cell sensitivity to a therapeutic agent are preferentially used. More preferentially, the suicide gene is the thymidine kinase gene. The expression product of this gene confers on mammalian cells sensitivity to certain therapeutic agents such as ganciclovir or acyclovir. Preferably the suicide gene is the herpes simplex virus thymidine kinase gene. The product of this is capable of phosphorylating nucleoside analogues such as acyclovir and ganciclovir. These modified molecules may be incorporated into a DNA chain

that is being extended, which has as consequence the stopping of DNA synthesis, causing the death of the cell (F.L. Moolten, Cancer Res. 46 (1986) 5276). This strategy thus makes it possible to specifically eliminate the cells expressing the suicide gene. Furthermore, the DNA synthesis being the target of the toxicity, only dividing cells are affected.

More preferentially, the human herpes virus thymidine kinase gene (hTK HSV-1) is used. The sequence of this gene has been described in the literature (see especially McKnight et al., Nucleic Acid. Res. 8 (1980) 5931). It is also possible to use derivatives of this sequence which have greater substrate specificity and a

better kinase activity. Such derivatives may be obtained, in particular, by mutagenesis at the level of the binding site as described above (Balasubramaniam et al., J. Gen. Virol. 71 (1990) 2979; Munir et al., JBC 267 (1992) 6584).

It is also possible to use the cytosine deaminase gene whose expression product confers on mammalian cells sensitivity to 5-fluorocytosine (5-FC) or nitroreductases which confer on mammalian cells sensitivity to the nitroaromatic products (J. Biol. Chem. 266 (1991) 4126).

As indicated above, it is most particularly advantageous to combine the suicide gene with an immunostimulant or tumour-suppressor gene. In this regard, the immunostimulant gene may be any gene whose expression product is capable of stimulating the body's defences. Preferentially, it is a gene encoding a cytokine, such as especially a lymphokine (IL-1 to IL-12), an interferon (alpha, beta and the like), a tumour necrosis factor, a colony-stimulating factor (G-CSF, GM-CSF, M-CSF, SCF and the like) and the like. Still more preferentially, it is the gene encoding interleukin-2 or GM-CSF. Interleukin-2 is synthesized essentially by the lymphocytes, in response to the presence of antigens, especially tumour antigens. It then acts on the development of the immune response against these antigens, in particular by local activation of the cytotoxic T and killer (NK) cells.

This lymphokine thus plays a major role in the antitumour immunity. By virtue of the vectors of the present invention, it is now possible to obtain a synergistic antitumour effect resulting from a simultaneous expression, in the same tumour cell, of interleukin-2 and of a suicide gene, such as the thymidine kinase gene.

GM-CSF is a granocyte and macrophage colony stimulating factor. It therefore stimulates the proliferation of these immune cells and therefore makes it possible to reinforce the immune defences. The GM-CSF gene and cDNA have been described in the literature. Its co-expression in a vector of the invention with a suicide gene produces a high synergistic antitumour effect.

Among the tumour-suppressor genes which may be used within the framework of the present invention, there may be mentioned more particularly the p53, Rb, rap 1A, DDC, WAF and MTS genes. More particularly, the p53 gene or the Rb gene is used.

The p53 gene encodes a nuclear protein of 53 kDa. The form of this gene which is mutated by deletion and/or mutation is involved in the development of most human cancers (Baker et al., Science 244 (1989) 217). Its mutated forms are also capable of cooperating with the ras oncogenes to transform murine fibroblasts. The wild-type gene encoding the native p53 inhibits, on the other hand, the formation of transformation foci in

rodent fibroblasts transfected with various combinations of oncogenes. Recent data emphasize that the p53 protein could itself be a transcription factor and could stimulate the expression of other tumour-suppressor genes. Moreover, an effect of p53 on the proliferation of vascular small muscle cells have been recently demonstrated (Epstein et al. Science 151 (1994)).

The Rb gene determines the synthesis of a nuclear phosphoprotein of about 927 amino acids (Friend et al., Nature 323 (1986) 643) whose function is to repress the division of cells by causing them to enter into a quiescence phase. Inactive forms of the Rb gene have been implicated in different tumours, and especially in retinoblastomas or in mesenchymatous cancers such as osteosarcomas. The reintroduction of this gene into the tumour cells where it was inactivated produces a return to the normal state and a loss of the tumorigenicity (Huang et al., Science 242 (1988) 1563). Recently, it was demonstrated that the normal Rb protein, but not its mutated forms, represses the expression of the proto-oncogene c-fos, a gene essential for cell proliferation.

The WAF and MTS genes and their antitumour properties have been described in the literature (Cell 75 (1993) 817; Science 264 (1994) 436).

In a particularly preferred embodiment, the invention relates to a defective recombinant adenovirus

comprising a gene encoding thymidine kinase and a tumour-suppressor gene. More preferentially, it relates to an adenovirus comprising a gene encoding the herpes virus thymidine kinase and the wild-type p53 gene (Ad-TK-p53). In a particularly advantageous manner, the two genes are placed under the control of separate promoters, preferably the HSV virus LTR promoter. Still more preferentially, the two genes are inserted at the level of the E1 region of the adenovirus genome.

In another particularly preferred embodiment, the invention relates to a defective recombinant adenovirus comprising a gene encoding thymidine kinase and a gene encoding a lymphokine. More preferentially, it relates to an adenovirus comprising a gene encoding the herpes virus thymidine kinase and a gene encoding interleukin-2 (Ad-TK-IL2) or GM-CSF (Ad-TK-GM-CSF). In a particularly advantageous manner, the two genes are placed under the control of separate promoters, preferably the HSV virus LTR promoter. Still more preferentially, the two genes are inserted at the level of the E1 region of the adenovirus genome.

As regards the transcriptional promoters used within the framework of the present invention, they may be promoters which are naturally responsible for the expression of the therapeutic gene considered when these are capable of functioning in the infected cell. They may be sequences of different origin (which are responsible for the expression of other proteins or

even synthetic). In particular, they may be promoter sequences of mammalian, eukaryotic or viral genes. For example, they may be promoter sequences derived from the genome of the cell which is desired to infect. Likewise, they may be promoter sequences derived from the genome of a virus, including the adenovirus used. In this regard, there may be mentioned for example the promoters of the E1A, MLP, CMV and RSV genes and the like. In addition, these expression sequences may be modified by the addition of activation and regulatory sequences, or of sequences allowing a tissue-specific expression. Moreover, when the inserted gene does not contain expression sequences, it may be inserted into the genome of the defective virus downstream of such a sequence. A preferred promoter for producing the vectors of the invention consists of the rous sarcoma virus LTR (LTR-RSV). Other particularly preferred promoters are the promoters which are specific for proliferative or cancer cells. These promoters indeed make it possible to target the therapeutic effect on a defined cell population.

In a preferred embodiment of the invention, the expression signals are induced by or are active in the presence of viruses responsible for or associated with tumours. Still more preferentially, an expression signal which is inducible by the Epstein-Barr virus (EBV) or by the papilloma virus is used within the framework of the present invention.

The Epstein-Barr virus (EBV) is associated with two types of human cancers: Burkitt's lymphoma and cancer of the nasopharynx. The use of a recombinant adenovirus comprising a toxic gene under the control of a promoter inducible by EBV advantageously makes it possible to specifically express this toxic gene in the tumour cells of the nasopharynx. In biopsies of cancers of the nasopharynx, a single nuclear antigen is regularly present, EBNA1, which is involved in maintaining the viral genome in the cells infected by EBV in the latent phase, and which transactivates the BCR2 viral promoter. A specific subject of the invention therefore consists in the use, for the specific expression of a gene in the cancer cells of the nasopharynx, of an EBNA1 responsive sequence (EBNA1-RE). In particular, the invention relates to an adenovirus comprising, as expression signal, a chimeric promoter comprising an EBNA1 responsive sequence used upstream of another viral promoter, the promoter of the gene for terminal protein 1 (TP1). The examples described in the present application show clearly that this chimeric promoter is inducible by EBNA1.

The papillomavirus (especially the HPV 16 and 18 viruses) are responsible for 90% of cervical cancers in women and have been identified in precancerous epithelial lesions (Riou et al., Lancet 335 (1990) 117). The product of the E6 gene leads to the formation of tumours by substantially decreasing the quantity of

wild-type p53, an anti-oncogene, in the HPV-positive cells (Wrede et al., Mol. Carcinog. 4 (1991) 171). The use of a recombinant adenovirus comprising a toxic gene under the control of a promoter inducible by HPV (for example the E6 protein) advantageously makes it possible to specifically express this toxic gene in the corresponding tumour cells.

The expression signals may further be inactive in normal cells and active in tumour cells. In particular, there may be used within the framework of the present invention the promoter of α-foetoprotein (Alpert E., dans Hepatocellular carcinoma. Okuda & Peters (eds), New York, 1976, 353) or the P3 promoter of IGF-II (Sussenbach et al., Growth Regulation 2 (1991) 1), which are active in adults, only in hepatocarcinomas. It is also possible to use promoters induced by hormones in the case of hormone-dependent or hormone-associated tumours (breast or prostate tumour for example).

As indicated above, different configurations may be envisaged for the production of the vectors of the invention. The vectors of the invention may first of all contain the two genes in the form of a single transcriptional entity. In this configuration, the two genes are contiguous, placed under the control of a single promoter, and give rise to a single premessenger RNA. This configuration is advantageous since it makes it possible to use a single transcriptional promoter to

regulate the expression of the 2 genes. Moreover, this single transcriptional entity may be incorporated into the adenoviral vector in the two possible orientations.

The two genes may also be placed under the control of separate transcriptional promoters. This configuration makes it possible to obtain higher expression levels and offers a better control of expression of the genes. In this case, the two therapeutic genes may be inserted in the same orientation or in opposite orientations.

The two genes may be inserted into the same site of the adenovirus genome or into different sites.

Preferentially, the genes are inserted, at least in part, at the level of the E1, E3 or E4 regions of the adenovirus genome. When they are inserted into two different sites, the use of the E1 and E3 or E1 and E4 regions is preferred within the framework of the invention. Preferably one of the genes is inserted at the level of the E1 region and the other at the level of the E3 or E4 region.

A particularly advantageous embodiment is that in which two therapeutic genes are inserted at the level of the E1 region. The examples show indeed that this organization allows a higher expression of the two genes, without interference between the two. The invention therefore also relates to any recombinant adenovirus comprising two therapeutic genes of interest inserted at the level of the E1 region of the genome.

Moreover, the immunostimulant gene may also comprise a signal sequence directing the product synthesized in the secretory pathways of the target cell. This signal sequence may be the natural signal

sequence of the immunostimulant product, but it may also be any other functional signal sequence or an artificial signal sequence.

As indicated above, the adenoviruses of the present invention are defective, that is to say that they are incapable of autonomously replicating in the target cell. Generally, the genome of the defective adenoviruses according to the present invention therefore lacks at least the sequences necessary for the replication of the said virus in the infected cell. These regions may be either eliminated (completely or in part), or made nonfunctional, or substituted by other sequences and especially by the therapeutic genes. The defective character of the adenoviruses of the invention is an important element, since it ensures the non-dissemination of the vectors of the invention after administration.

In a preferred embodiment, the adenoviruses of the invention comprise the ITR sequences and a sequence allowing the encapsidation, and possess a deletion of all or part of the E1 gene.

The inverted repeat sequences (ITR) constitute the replication origin of the adenoviruses. They are located between the 3' and 5' ends of the viral genome (cf. Figure 1), from where they can be easily isolated according to conventional molecular biology techniques known to persons skilled in the art. The nucleotide sequence of the ITR sequences of the

human adenoviruses (in particular of the Ad2 and Ad5 serotypes) is described in the literature, as well as of the canine adenoviruses (especially CAV1 and CAV2). As regards the Ad5 adenovirus for example, the left-hand ITR sequence corresponds to the region comprising nucleotides 1 to 103 of the genome.

The encapsidation sequence (also designated Psi sequence) is necessary for the encapsidation of the viral DNA. This region must therefore be present to allow the preparation of defective recombinant adenoviruses according to the invention. The encapsidation sequence is located in the adenovirus genome, between the left-hand (5') ITR and the E1 gene (cf. Figure 1). It may be isolated or synthesized artificially by conventional molecular biology techniques. The nucleotide sequence of the encapsidation sequence of human adenoviruses (in particular of the Ad2 and Ad5 serotypes) is described in the literature, as well as of the canine adenoviruses (especialy CAV1 and CAV2). As regards the Ad5 adenovirus for example, the encapsidation sequence corresponds to the region comprising nucleotides 194 to 358 of the genome.

More preferentially, the adenoviruses of the invention comprise the ITR sequences and a sequence allowing encapsidation, and having a deletion of all or part of the E1 and E4 genes.

In a particularly preferred embodiment, the

genome of the adenoviruses according to the invention is deleted of all or part of the E1, E3 and E4 genes and, still more preferentially, of all or part of the E1, E3, L5 and E4 genes.

The adenoviruses of the invention may be prepared from adenoviruses of diverse origins. There are indeed different adenovirus serotypes, whose structure and properties vary somewhat, but which exhibit a comparable genetic organization. Thus, the teachings described in the present application may be easily reproduced by persons skilled in the art for any type of adenovirus.

More particularly, the adenoviruses of the invention may be of human, animal or mixed (human and animal) origin.

As regards the adenoviruses of human origin, the use of those classified in group C is preferred. More preferentially, among the different human adenovirus serotypes, the use of the type 2 or 5 adenoviruses (Ad 2 or Ad 5) is preferred within the framework of the present invention.

As indicated above, the adenoviruses of the invention may also be of animal origin, or may comprise sequences derived from adenoviruses of animal origin. The Applicant has indeed shown that the adenoviruses of animal origin are capable of infecting human cells with a high efficiency, and that they are incapable of propagating in the human cells in which they have been

tested (cf. Application FR 93 05954). The Applicant has also shown that the adenoviruses of animal origin are not at all trans-complemented by adenoviruses of human origin, which removes any risk of recombination and of propagation in vivo, in the presence of a human adenovirus, capable of leading to the formation of an infectious particle. The use of adenoviruses or of regions of adenoviruses of animal origin is therefore particularly advantageous since the risks inherent in the use of viruses as vectors in gene therapy are even smaller.

The adenoviruses of animal origin which can be used within the framework of the present invention may be canine, bovine, murine (example: Mav1, Beard et al., Virology 75 (1990) 81), ovine, porcine, avian or simian (example: SAV) origin. More particularly, among the avian adenoviruses, there may be mentioned the serotypes 1 to 10 which are accessible at ATCC, such as for example the strains Phelps (ATCC VR-432), Fontes (ATCC VR-280), P7-A (ATCC VR-827), IBH-2A (ATCC VR-828), J2-A (ATCC VR-829), T8-A (ATCC VR-830), K-11 (ATCC VR-921) or the reference strains ATCC VR-831 to 835. Among the bovine adenoviruses, there may be used the different serotypes known, and especially those available at the ATCC (types 1 to 8) under the references ATCC VR-313, 314, 639-642, 768 and 769. There may also be mentioned the murine adenoviruses FL (ATCC VR-550) and E20308 (ATCC VR-528), the ovine

adenovirus type 5 (ATCC VR-1343) or type 6 (ATCC R-1340); the porcine adenovirus 5359), or the simian adenoviruses such as especially the adenoviruses referenced at the ATCC under the numbers VR-591-594, 941-943, 195-203 and the like.

Preferably, among the different adenoviruses of animal origin, adenoviruses or regions of adenoviruses of canine origin, and especially all the CAV2 adenovirus strains [Manhattan or A26/61 strain (ATCC VR-800) for example] are used within the framework of the invention. The canine adenoviruses have been the subject of numerous structural studies. Thus, complete restriction maps of the CAV1 and CAV2 adenoviruses have been described in the prior art (Spibey et al., J. Gen. Virol. 70 (1989) 165), and the E1a and E3 genes as well as the ITR sequences have been cloned and sequenced (see especially Spibey et al., Virus Res. 14 (1989) 241; Linné, Virus Res. 23 (1992) 119, WO 91/11525).

The defective recombinant adenoviruses according to the invention may be prepared in various ways.

A first method consists in transfecting the DNA of the defective recombinant virus prepared in vitro (either by ligation or in plasmid form) into a competent cell line, that is to say carrying in trans all the functions necessary for the complementation of the defective virus. These functions are preferentially

integrated into the genome of the cell, which makes it possible to avoid the risks of recombination, and confers an increased stability on the cell line.

A second approach consists in co-transfecting into an appropriate cell line the DNA from the defective recombinant virus prepared in vitro (either by ligation or in plasmid form) and the DNA from a helper virus. According to this method, it is not necessary to have a competent cell line capable of complementing all the defective functions of the recombinant adenovirus. A part of these functions is indeed complemented by the helper virus. This helper virus should itself be defective and the cell line carries in trans the functions necessary for its complementation. Among the cell lines which may be used especially in the context of this second approach, there may be mentioned especially the human embryonic line 293, the KB cells, the cells Hela, MDCK, GHK and the like (cf. Examples).

Next, the vectors which have multiplied are recovered, purified and amplified according to conventional molecular biology techniques.

According to a variant embodiment, it is possible to prepare in vitro, either by ligation or in plasmid form, the defective recombinant virus DNA carrying the appropriate deletions and the two therapeutic genes. As indicated above, the vectors of the invention advantageously possess a deletion of all

or part of certain viral genes, especially the E1, E3, E4 and/or L5 genes. This deletion may correspond to any type of suppression affecting the gene considered. It may be especially a suppression of all or part of the coding region of the said gene, and/or of all or part of the transcriptional promoter region of the said gene. The suppression is generally produced on the defective recombinant viral DNA, for example by digestion by means of appropriate restriction enzymes, then ligation, according to molecular biology techniques, as illustrated in the Examples. The therapeutic genes may then be inserted into this DNA by enzymatic cleavage and then ligation, at the level of the regions selected and in the chosen orientation.

The DNA thus obtained, which therefore carries the appropriate deletions and the two therapeutic genes, makes it possible to directly generate the defective recombinant adenovirus carrying the said deletions and therapeutic genes. This first variant is particularly adapted to the production of recombinant adenoviruses in which the therapeutic genes are arranged in the form of a single transcriptional unit or under the control of separate promoters but which are inserted at the same site of the genome.

It is also possible to prepare the recombinant virus in two stages, allowing the successive introduction of the two therapeutic genes. Thus, the DNA from a first recombinant virus carrying

the appropriate deletions (or part of the said deletions) and one of the therapeutic genes is constructed, by ligation or in plasmid form. This DNA is then used to generate a first recombinant virus carrying the said deletions and a therapeutic gene. The DNA from this first virus is then isolated and co-transfected with a second plasmid or the DNA from a second defective recombinant virus carrying the second therapeutic gene, the appropriate deletions (part not present on the first virus), and a region allowing homologous recombination. This second stage thus generates the defective recombinant virus carrying the two therapeutic genes. This variant preparation is particularly appropriate for the preparation of recombinant viruses carrying two therapeutic genes inserted into two different regions of the adenovirus genome.

The present invention also provides a pharmaceutical composition comprising at least one defective recombinant adenovirus as described above and a pharmaceutically acceptable vehicle. The pharmaceutical compositions of the invention may be formulated for administration via the topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular or transdermal route.

Preferentially, the pharmaceutical composition contains pharmaceutically acceptable vehicles for an injectable formulation. They may be in

particular isotonic sterile saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like, or mixtures of such salts), or dry, especially freeze-dried compositions which, upon addition, depending on the case, of sterilized water or of physiological saline, allow the preparation of injectable solutions.

The virus doses used for the injection may be adapted according to different parameters, and especially according to the mode of administration used, the relevant pathology, the gene to be expressed, or the desired duration of treatment. In general, the recombinant adenoviruses according to the invention are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu/ml, and preferably 10^6 to 10^{10} pfu/ml. The term pfu ("plaque forming unit") corresponds to the infectivity of a virus solution, and is determined by infecting an appropriate cell culture and measuring, generally after 5 days, the number of infected cell plaques. The techniques for determining the pfu titre of a viral solution are well documented in the literature.

The adenoviruses of the invention may be used for the treatment or prevention of numerous pathologies. They are particularly advantageous for the treatment of hyperproliferative pathologies (cancers, restenosis and the like), by direct injection at the level of the relevant site. In this regard, the present

invention also relates to a method for the destruction of proliferative cells comprising the infection of the said cells or a portion of them with an adenoviral vector as defined above. In the case where the suicide gene is a gene conferring sensitivity to a therapeutic agent, the method of destruction according to the invention then comprises the treatment of the cells with the said therapeutic agent. For the implementation of this method, the invention also provides a product comprising:

- one or more recombinant adenoviruses as defined above in which the suicide gene is a gene conferring sensitivity to a therapeutic agent, and
- the said therapeutic agent

as combination product for a simultaneous or separate use or for a use separated out over time, for the treatment of hyperproliferative pathologies. More particularly, the suicide gene is a thymidine kinase gene and the therapeutic agent is gancyclovir or acyclovir or an analogue.

The present invention will be more fully described with the aid of the following examples which should be considered as illustrative and non-limiting.

Legend to the figures

Figure 1: Genetic organization of the Ad5 adenovirus. The complete sequence of Ad5 is available on database and allows persons skilled in the art to select or create any restriction site, and thus to isolate any region of the genome.

Figure 2: Restriction map of the CAV2 adenovirus,

Manhattan strain (according to Spibey et al., cited above).

Figure 3: Representation of the vector pONTtk

Figure 4: Representation of the vector pRSVtk

General molecular biology techniques

The methods conventionally used in molecular biology, such as preparative extractions of plasmid DNA, centrifugation of plasmid DNA in a caesium chloride gradient, agarose or acrylamide gel electrophoresis, purification of DNA fragments by electroelution, phenol or phenol-chloroform extraction of proteins, ethanol or isopropanol precipitation of DNA in saline medium, transformation in Escherichia coli and the like, are well known to persons skilled in the art and are abundantly described in the literature [Maniatis T. et al., "Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel F.M. et al. (eds), "Current Protocols in Molecular Biology", John Wiley & Sons, New York, 1987].

The plasmids of the pBR322 and pUC types and the phages of the M13 series are of commercial origin (Bethesda Research Laboratories).

For the ligations, the DNA fragments may be separated according to their size by agarose or acrylamide gel electrophoresis, extracted with phenol or with a phenol/chloroform mixture, precipitated with ethanol and then incubated in the presence of T4 phage

DNA ligase (Biolabs) according to the recommendations of the supplier.

The filling of the protruding 5' ends may be carried out with the Klenow fragment of DNA polymerase I of E. coli (Biolabs) according to the supplier's specifications. The destruction of the protruding 3' ends is carried out in the presence of T4 phage DNA polymerase (Biolabs) used according to the recommendations of the manufacturer. The destruction of the protruding 5' ends is carried out by a controlled treatment with S1 nuclease.

The in vitro synthetic oligodeoxynucleotide-directed mutagenesis may be carried out according to the method developed by Taylor et al. [Nucleic Acids Res. 13 (1985) 8749-8764] using the kit distributed by Amersham.

The enzymatic amplification of the DNA fragments by the so-called PCR [Polymerase-catalyzed Chain Reaction, Saiki R.K. et al., Science 230 (1985) 1350-1354; Mullis K.B. et Faloona F.A., Meth. Enzym. 155 (1987) 335-350] technique may be carried out using a "DNA thermal cycler" (Perkin Elmer Cetus) according to the manufacturer's specifications.

The verification of the nucleotide sequences may be carried out by the method developed by Sanger et al. [Proc. Natl. Acad. Sci. USA, 74 (1977) 5463-5467] using the kit distributed by Amersham.

Cell lines used

In the following examples, the following cell lines were or may be used:

- Human embryonic kidney line 293 (Graham et al., J. Gen. Virol. 36 (1977) 59). This line contains especially, integrated into its genome, the left-hand part of the genome of the human adenovirus Ad5 (12%).

- KB human cell line: derived from a human epidermal carcinoma, this line is accessible at the ATCC (ref. CCL 17) as well as the conditions allowing its culture.

- Hela human cell line: derived from a human epithelial carcinoma, this line is accessible at the ATCC (ref. CCL2) as well as the conditions allowing its culture.

- MDCK canine cell line: the conditions of culture of the MDCK cells have been described especially by Macatney et al., Science 44 (1988) 9.

- The gm DBP6 cell line (Brough et al., Virology 190 (1992) 624). This line consists of Hela cells carrying the adenovirus E2 gene under the control of the MMTV LTR.

EXAMPLES

Example 1. Construction of defective recombinant adenoviruses comprising the TK gene under the control of a cancer-specific promoter and the p53 gene under the control of the CMV promoter.

These adenoviruses were constructed by homologous recombination between a plasmid carrying the left-hand part of the Ad5 adenovirus, the two therapeutic genes and a region of the Ad5 adenovirus (corresponding to the IX protein) and the DNA from a defective adenovirus carrying different deletions.

1. Construction of the vector pONT-tk

1.1. Construction of the plasmid p7tk1

This example describes the construction of the plasmid p7tk1 containing the open reading frame of the tk gene of 1131 base pairs (ATG 114-116 and stop codon TGA 1242-1244), inserted into a multiple cloning site.

The BgIII-NcoI fragment containing the herpes simplex virus type 1 thymidine kinase (tk) gene was isolated from plasmid pHSV-106 (marketed by Gibco BRL), repaired by the action of the klenow fragment and then inserted into the SmaI site of the plasmid pGEM7zf(+) (marketed by Promega). The SmaI and BgIII sites are destroyed during this step, the NcoI site is conserved.

The plasmid obtained was designated p7tk1.

1.2. Construction of the plasmid pONT1

This example describes the construction of a plasmid containing a chimeric promoter consisting of a sequence necessary for the transactivation of the EBNA1 antigen and of the TP1 promoter of the EBV virus.

The EcoRI(7315)-SmaI (8191) fragment of the EBV virus was isolated from the strain B95-8. The

complete sequence of the EBV virus has been described by Baer et al. (Nature 310 (1984) 207). This fragment contains the sequences necessary for the trans-activation by the nuclear antigen 1 (EBNA1) (D. Reisman & B. Sugden, 1986, Molecular and Cellular Biology, vol. 6 pp. 3838-3846). This fragment was then fused to the NruI(166 241)-PstI(166 559) fragment of EBV B95-8 (the PstI site was digested with T4 polymerase), containing the TP1 promoter. The chimeric promoter thus obtained was then inserted into the multiple cloning site of the plasmid pBluescript II SK.

The plasmid obtained was designated pONT1.

1.3. Construction of the plasmid pONTtk

The plasmid pONTtk comprises the herpes simplex virus thymidine kinase (tk) gene cloned into the plasmid p7tk1, under the control of the chimeric promoter EBNA1-RE/TP1 cloned into the plasmid pONT1.

To construct this plasmid, the BamHI-XhoI fragment of pONT1 which contains the chimeric promoter transactivated by EBNA-1 and EBNA-2, and the XhoI-ClaI fragment of p7tk1 which contains the tk open reading frame were cloned into the BamHI (478) and ClaI (4550) sites of the plasmid pAd.RSVβgal. The plasmid pAd.RSVβGal contains, in the 5'→3' orientation,

- the PvuII fragment corresponding to the left-hand end of the Ad5 adenovirus comprising: the ITR sequence, the replication origin, the encapsidation and enhancer signals E1A;

- the gene encoding β-galactosidase under the control of the RSV promoter (Rous sarcoma virus),

- a second fragment of the Ad5 adenovirus genome which allows the homologous recombination between the plasmid pAd.RSVβGal and the adenovirus dl324. The plasmid pAd.RSVβGal has been described by Stratford-Perricaudet et al. (J. Clin. Invest. 90 (1992) 626).

All the cloning sites are conserved. The plasmid obtained was designated pONTtk (Figure 3).

2. Construction of the plasmid pONTtkCMVp53

This example describes the construction of a vector carrying the left-hand part of the Ad5 adenovirus (comprising the left-hand ITR, the encapsidation region and the start of the E1 region), the tk gene under the control of the ONT promoter, the p53 gene under the control of the CMV promoter and a second fragment of the Ad5 genome (pIX protein) allowing homologous recombination for the generation of the recombinant adenovirus (cf. Example 1.3.).

This plasmid was constructed from the plasmid pONTtk by insertion, downstream of the tk gene and in the same orientation, of a fragment carrying the p53 gene under the control of the CMV promoter and followed by the polyadenylation site of the SV40 virus. More specifically, the inserted fragment comprises:

- a promoter region of viral origin which corresponds to the cytomegalovirus (CMV) early

promoter. This region is surrounded, in the vector, by unique restriction sites EcoRI-SphI at the CMV/pONTtk junction and BamHI at the CMV/p53 junction. The presence of unique sites flanking the promoter region makes it possible to replace the CMV region by any other promoter. A second series of vectors is thus obtained in which the p53 gene is placed under the control of an inducible promoter: the metallothionin promoter, inducible by heavy metals (cadnium and zinc).

- A 1173 bp sequence corresponding to the cDNA encoding the mouse p53 protein in its wild-type form (Zakut-Houri et al., Nature 36 (1983) 594). In this construct, the suppressor gene is in the form of cDNA, that is to say lacks introns. This makes it possible especially to reduce the size of the vector. Moreover, it was checked that the expression levels obtained are comparable in the presence or in the absence of introns.

- The polyadenylation signal of the SV40 virus late genes, which corresponds to a very efficient polyadenylation signal. Two unique restriction sites SalI and HindIII are situated downstream of the polyadenylation signal.

The vector obtained was designated pONTtkCMVp53.

It is understood that the insertion of the said fragment may be made in a similar manner in the opposite orientation, leading to a plasmid in which the

tk gene and the p53 gene are in opposite orientations (pONTtkCMVp53inv).

3. Construction of the recombinant adenoviruses

3.1. Construction of a recombinant adenovirus deleted in the E1 region, carrying the two therapeutic genes inserted in the same orientation, at the level of the E1 region.

The vector pONTtkCMVp53 was linearized and co-transfected with an adenoviral vector deficient in the E1 gene, into the helper cells (line 293) providing in *trans* the functions encoded by the adenovirus E1 (E1A and E1B) regions.

More precisely, the adenovirus Ad-ONTtkCMVp53,ΔE1 is obtained by homologous recombination in vivo between the adenovirus Ad-RSVβgal (cf. Stratford-Perricaudet et al., cited above) and the vector pONTtkCMVp53, according to the following procedure: the plasmid pONTtkCMVp53, linearized by XmnI, and the adenovirus Ad-RSVβgal, linearized by the ClaI enzyme, are co-transfected into the line 293 in the presence of calcium phosphate, so as to allow the homologous recombination. The recombinant adenoviruses thus generated are then selected by plaque purification. After isolation, the recombinant adenovirus DNA is amplified in the 293 cell line, which leads to a culture supernatant containing the unpurified recombinant defective adenovirus having a

titre of about 10^{10} pfu/ml.

The viral particles are generally purified by centrifugation on a caesium chloride gradient according to known techniques (see especially Graham et al., Virology 52 (1973) 456). The adenovirus Ad-ONT-tkCMVp53,ΔE1 may be stored at -80°C (in 20% glycerol).

3.2. Construction of a recombinant adenovirus deleted in the E1 and E3 regions, carrying the two therapeutic genes inserted in the same orientation, at the level of the E1 region.

The vector pONTtkCMVp53 was linearized and co-transfected with an adenoviral vector deficient in the E1 and E3 genes into the helper cells (line 293) providing in *trans* the functions encoded by the adenovirus E1 (E1A and E1B) regions.

More precisely, the adenovirus ad-ONTtkCMVp53,ΔE1,ΔE3 is obtained by homologous recombination in vivo between the mutant adenovirus Ad-dl1324 (Thimmappaya et al., Cell 31 (1982) 543) and the vector pONTtkCMVp53, according to the following procedure: the plasmid pONTtkCMVp53, linearized with XmnI and the adenovirus Ad-dl1324, linearized with the enzyme ClaI, are co-transfected into the line 293 in the presence of calcium phosphate, so as to allow the homologous recombination. The recombinant adenoviruses thus generated are then selected by plaque purification. After isolation, the recombinant adenovirus DNA is amplified in the 293 cell line, which

leads to a culture supernatant containing the unpurified recombinant defective adenovirus having a titre of about 10^{10} pfu/ml.

The viral particles are generally purified by centrifugation on a caesium chloride gradient according to known techniques (see especially Graham et al., Virology 52 (1973) 456). The adenovirus Ad-ONT-tkCMVp53,ΔE1,ΔE3 may be stored at -80°C in 20% glycerol.

3.3. Construction of adenoviruses in which the tk and p53 genes are positioned in opposite orientations.

By following the procedures described in Examples 3.1. and 3.2. above, adenoviruses in which the tk and p53 genes are positioned in opposite orientations may be constructed starting with the plasmid pONTtkCMVp53inv.

<u>Example 2.</u> Construction of defective recombinant adenoviruses comprising the TK gene under the control of the RSV virus LTR promoter and the p53 gene under the control of the CMV promoter.

These adenoviruses were constructed by homologous recombination between a plasmid carrying the left-hand part of the Ad5 adenovirus, the two therapeutic genes and a region of the Ad5 adenovirus (corresponding to the IX protein) and the DNA from a defective adenovirus carrying different deletions.

1. Construction of the vector pRSVtk

This plasmid was constructed from the plasmid pONTtk (Example 1.1), by substitution of the EBNA1-transactivable promoter by the RSV LTR promoter. For that, the RSV promoter was isolated in the form of a BamHI-SalI fragment from the plasmid pAd.RSV.βgal (Stratford-Perricaudet et al., J. Clin. Invest. 90 (1992) 626), and then cloned into the BamHI(478) and SalI(1700) sites of the plasmid pONTtk. The resulting plasmid was designated pRSVtk (Figure 4).

2. Construction of the plasmid pRSVtkCMVp53

This example describes the construction of a vector carrying the left-hand part of the Ad5 adenovirus (comprising the left-hand ITR, the encapsidation region and the start of the E1 region), the tk gene under the control of the RSV promoter, the p53 gene under the control of the CMV promoter and a second fragment of the Ad5 genome (pIX protein) allowing homologous recombination for the generation of the recombinant adenovirus (cf. Example 2.3.).

This plasmid was constructed from the plasmid pRSVtk by insertion, downstream of the tk gene of a fragment carrying the p53 gene under the control of the CMV promoter and followed by the polyadenylation site of the SV40 virus. More specifically, the inserted fragment comprises:

- a promoter region of viral origin which corresponds to the cytomegalovirus (CMV) early

promoter. This region is surrounded, in the vector, by unique restriction sites EcoRI-SphI at the CMV/pONTtk junction and BamHI at the CMV/p53 junction. The presence of unique sites flanking the promoter region makes it possible to replace the CMV region by any other promoter. A second series of vectors is thus obtained in which the p53 gene is placed under the control of an inducible promoter: the metallothionin promoter, inducible by heavy metals (cadnium and zinc).

- A 1173 bp sequence corresponding to the cDNA encoding the mouse p53 protein in its wild-type form (Zakut-Houri et al., Nature 36 (1983) 594). In this construct, the suppressor gene is in the form of cDNA, that is to say lacks introns. This makes it possible especially to reduce the size of the vector. Moreover, it was checked that the expression levels obtained are comparable in the presence or in the absence of introns.

- The polyadenylation signal of the SV40 virus late genes, which corresponds to a very efficient polyadenylation signal. Two unique restriction sites SalI and HindIII are situated downstream of the polyadenylation signal.

The vector obtained was designated pRSVtkCMVp53.

3. Construction of the recombinant adenoviruses

3.1. Construction of a recombinant adenovirus deleted in the E1 region, carrying the two therapeutic genes inserted in the same orientation, at the level of the E1 region.

This adenovirus is constructed according to the procedure described in Example 1(3.1.). The adenovirus Ad-RSV-tkCMVp53,ΔE1 thus obtained may be stored at -80°C in 20% glycerol.

3.2. Construction of a recombinant adenovirus deleted in the E1 and E3 regions, carrying the two therapeutic genes inserted in the same orientation, at the level of the E1 region.

This adenovirus is constructed according to the procedure described in Example 1(3.2.). The adenovirus Ad-RSV-tkCMVp53,ΔE1,ΔE3 thus obtained may be stored at -80°C in 20% glycerol.

Example 3. **Construction of defective recombinant adenoviruses comprising the TK gene under the control of the RSV virus LTR promoter and the interleukin-2 gene under the control of the same promoter.**

These adenoviruses were constructed by homologous recombination between a plasmid carrying the left-hand part of the Ad5 adenovirus, the two therapeutic genes and a region of the Ad5 adenovirus (corresponding to the IX protein) and the DNA from a defective adenovirus carrying different deletions.

1. Construction of the plasmid pRSVtkRSVIL-2

This example describes the construction of a vector carrying the left-hand part of the Ad5 adenovirus (comprising the left-hand ITR, the encapsidation region and the start of the El region), the tk gene under the control of the RSV promoter, the interleukin-2 gene under the control of the RSV promoter and a second fragment of the Ad5 genome (pIX protein) allowing homologous recombination for the generation of the recombinant adenovirus (cf. Example 3.2.).

This plasmid was constructed from the plasmid pRSVtk (cf Example 2) by insertion, downstream of the tk gene, of a fragment carrying the interleukin-2 gene under the control of the RSV promoter and followed by the SV40 virus polyadenylation site. More precisely, the fragment inserted comprises:

- the RSV virus LTR promoter, isolated in the form of a BamHI-SalI fragment from the plasmid pAd.RSV.βgal (Stratford-Perricaudet et al., J. Clin. Invest. 90 (1992) 626);

- a sequence corresponding to the cDNA encoding human interleukin-2 (EP91 539). In this construct, the therapeutic gene is in the form of cDNA.

- The polyadenylation signal of the SV40 virus late genes, which corresponds to a very efficient polyadenylation signal. Two unique SalI and HindIII restriction sites are situated downstream of the

polyadenylation signal.

The vector obtained was designated pRSVtkRSVIL-2.

2. Construction of the recombinant adenoviruses

Two types of recombinant adenoviruses are constructed according to the procedure described in Example 1 or 2. These adenoviruses carry the two therapeutic genes inserted in the same orientation, at the level of the E1 region and have a deletion in the E1 region or in the E1 and E3 regions.

The adenoviruses Ad-RSV-tkRSVIL-2,ΔE1 and Ad-RSV-tkRSVIL-2,ΔE1,ΔE3 thus obtained may be stored at -80°C in 20% glycerol.

Example 4. Construction of defective recombinant adenoviruses comprising the TK gene and the gene encoding the granocyte and macrophage colony stimulating factor (GM-CSF).

By following the procedures described in the preceding examples, defective adenoviruses carrying the tk gene (under the control of the ONT or RSV promoter for example) and the GM-CSF gene under the control of its own promoter or of the RSV promoter in particular may be constructed.

For this, an intermediate vector carrying the two genes may be constructed from the vector pONTtk or pRSVtk by insertion, in the same orientation or in the opposite orientation, of a fragment carrying the GM-CSF

gene under the control of the promoter. The gene encoding GM-CSF and constructs containing it have been described especially in Application WO 86/03225.

<u>Example 5.</u> Construction of defective recombinant adenoviruses comprising two genes of interest, one inserted at the level of the E1 region and the other inserted at the level of the E3 region.

These adenoviruses are constructed by homologous recombination between a DNA of a first defective virus carrying the first gene inserted at the level of the E1 region and the DNA of a second defective adenovirus carrying the second gene inserted at the level of the E3 region.

<u>1. Construction of the defective virus carrying the gene inserted at the level of the E3 region</u>

This virus is constructed from the adenovirus Addl324 (Thimmappaya et al., Cell 31 (1982) 543). This virus carries a deletion at the level of the E1 and E3 regions (XbaI-EcoRI fragment deleted). The Addl324 virus DNA was isolated and purified. This DNA is then cut with the enzymes XbaI and EcoRI. An XbaI-EcoRI fragment is then derived from the plasmid pRSVtk carrying the sequence encoding thymidine kinase under the control of the RSV promoter, and then inserted at the level of the said sites in the open Addl324 DNA as above.

The DNA thus obtained therefore comprises a

deletion at the level of the E1 region and the TK gene inserted at the level of the E3 region.

2. Construction of the adenoviruses carrying the two genes.

The DNA from the recombinant virus prepared above and the DNA from a recombinant adenovirus carrying an immunostimulant or tumour-suppressor gene inserted at the level of the E1 region, linearized by BamHI, are co-transfected into the line 293 in the presence of calcium phosphate, so as to allow the homologous recombination. The recombinant adenoviruses thus generated are then selected by plaque purification. After isolation, the recombinant adenovirus DNA is amplified in the 293 cell line, which leads to a culture supernatant containing the unpurified recombinant defective adenovirus having a titre of about 10^{10} pfu/ml.

The viral particles are generally purified by centrifugation on a caesium chloride gradient according to known techniques (see especially Graham et al., Virology 52 (1973) 456).

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Defective recombinant adenovirus characterized in that it comprises two therapeutic genes, the first being a suicide gene and the second an immunostimulant gene or a tumour-suppressor gene.

2. Adenovirus according to Claim 1, characterized in that the two genes constitute a single transcriptional entity under the control of a single promoter.

3. Adenovirus according to Claim 1, characterized in that the two genes are under the control of separate transcriptional promoters.

4. Adenovirus according to Claim 3, characterized in that the two genes are inserted in the same orientation.

5. Adenovirus according to Claim 3, characterized in that the two genes are inserted in opposite orientations.

6. Adenovirus according to any one of Claims 1 to 5, characterized in that the two therapeutic genes are inserted into the same site of the genome.

7. Adenovirus according to Claim 6 wherein the two genes are inserted at the level of the E1, E3 or E4 regions.

8. Adenovirus according to Claim 6, characterized in that the two genes are inserted at the level of the E1 region.

9. Adenovirus according to any one of Claims 1, 3, 4 and 5, characterized in that the two therapeutic genes are inserted into different sites of the genome.

10. Adenovirus according to Claim 9, characterized in that one of the genes is inserted at the level of the E1 region and the other at the level of the E3 or E4 region.

11. Adenovirus according to any one of Claims 1 to 10, characterized in that it comprises the ITR sequences, a sequence allowing encapsidation, and in that it carries a deletion of all or part of the E1 and E4 genes.

12. Adenovirus according to Claim 11, characterized in that it comprises the ITR sequences, a sequence allowing encapsidation, and in that it carries a deletion of all or part of the E1, E3 and E4 genes.

13. Adenovirus according to any one of Claims 1 to 12, characterized in that its genome is deleted of all or part of the E1, E3, L5 and E4 genes.

14. Adenovirus according to Claim 1, characterized in that it is of human, animal or mixed origin.

15. Adenovirus according to Claim 14, characterized in that the adenoviruses of human origin are chosen from those classified in group C.

16. Adenovirus according to Claim 15 which is a type 2 or 5 adenovirus (Ad 2 or Ad 5).

17. Adenovirus according to Claim 14, characterized in that the adenoviruses of animal origin are chosen from the adenoviruses of canine, bovine, murine, ovine, porcine, avian and simian origin.

18. Adenovirus according to Claim 1, characterized in that the suicide gene is a thymidine kinase gene.

19. Adenovirus according to Claim 18, wherein the suicide gene is the herpes virus HSV-I thymidine kinase gene.

20. Adenovirus according to Claim 1, characterized in that it comprises a gene encoding thymidine kinase and a tumour-suppressor gene.

21. Adenovirus according to Claim 1, characterized in that it comprises a gene encoding thymidine kinase and a gene encoding a lymphokine.

22. Defective recombinant adenovirus characterized in that it comprises a gene encoding the herpes virus

thymidine kinase and the wild-type p53 gene (Ad-TK-p53).

23. Defective recombinant adenovirus characterized in that it comprises a gene encoding the herpes virus thymidine kinase and a gene encoding interleukin-2 (Ad-TK-IL2).

24. Defective recombinant adenovirus characterized in that it comprises a gene encoding the herpes virus thymidine kinase and a gene encoding GM-CSF (Ad-TK-GM-CSF).

25. Adenovirus according to Claim 2 or 3, characterized in that the transcriptional promoter(s) are chosen from mammalian, eukaryotic or viral promoters.

26. Adenovirus according to any one of the preceding claims, characterized in that the immunostimulant gene comprises a signal sequence directing the therapeutic product synthesized in the secretory pathways of the target cell.

27. Pharmaceutical composition comprising at least one defective recombinant adenovirus according to one of Claims 1 to 25 and a pharmaceutically acceptable vehicle.

28. Pharmaceutical composition according to Claim 27, comprising a pharmaceutically acceptable vehicle for an injectable formulation.

29. Product comprising:

- one or more recombinant adenoviruses as defined in any one of Claims 1 to 26 in which the suicide gene is a gene conferring sensitivity to a therapeutic agent, and

- the said therapeutic agent

as combination product for a simultaneous or separate use or for a use spread out over time for the treatment of hyperproliferative pathologies.

30. Product according to Claim 29, characterized in that the suicide gene is a thymidine kinase gene and the therapeutic agent is gancyclovir or acyclovir or an

analogue.

31. Adenovirus according to Claim 1 substantially as hereinbefore described in any one of the Examples.

32. Pharmaceutical composition according to Claim 27 substantially as hereinbefore described in any one of the Examples.

33. Product according to Claim 29 substantially as hereinbefore described in any one of the Examples.

DAted this 15th day of Novembe 1999
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